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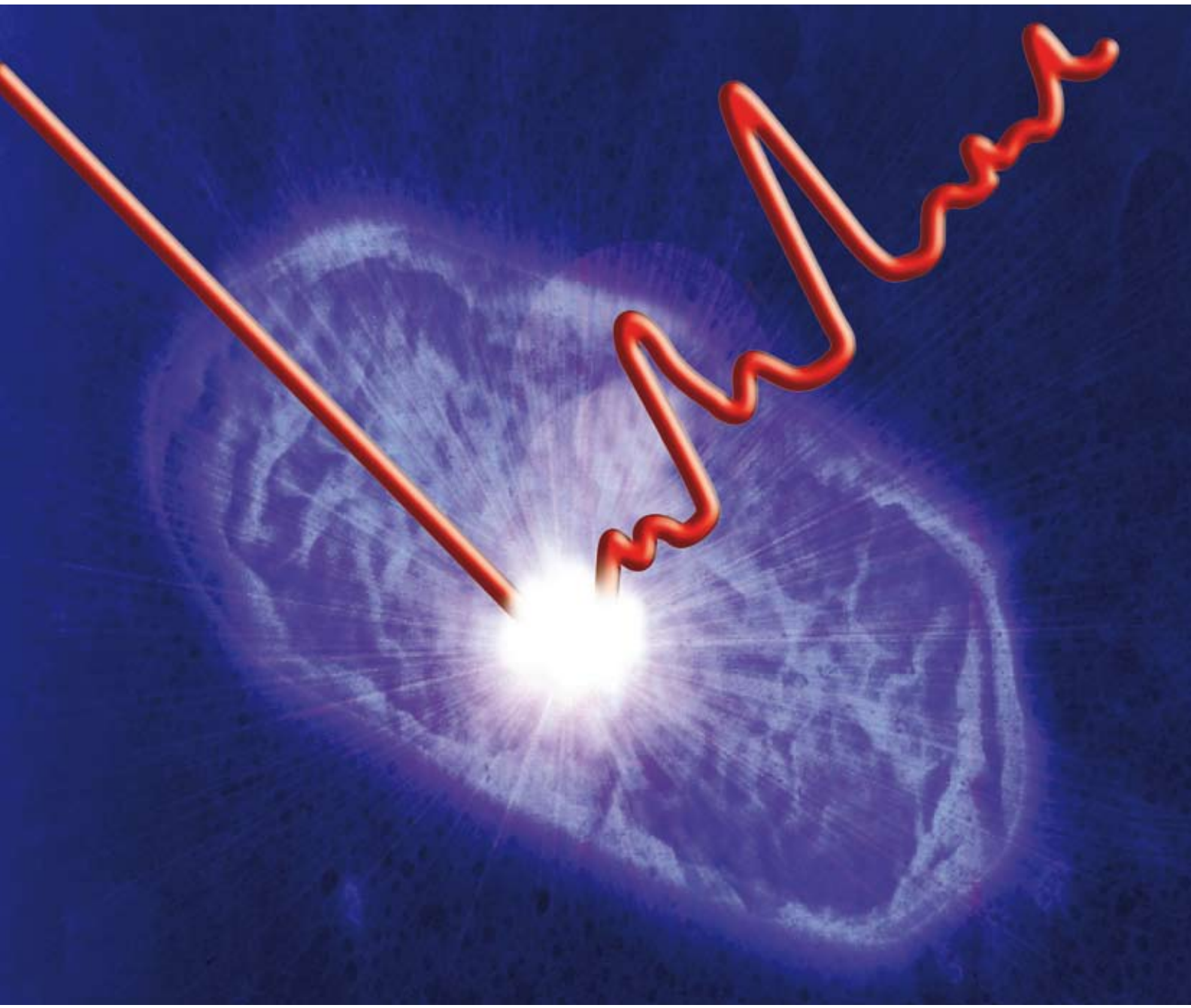

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Characterisation and identification of bacteria using SERS

Roger M. Jarvis* and Royston Goodacre

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Within microbiology Raman spectroscopy is considered as a very important whole-organism fingerprinting technique, which is used to characterise, discriminate and identify microorganisms and assess how they respond to abiotic or biotic stress. Enhancing the sensitivity of Raman spectroscopy is very beneficial for the rapid analysis of bacteria (and indeed biological systems in general), where the ultimate goal is to achieve this without the need for lengthy cell culture. Bypassing this step would provide significant benefits in many areas such as medical, environmental and industrial microbiology, microbial systems biology, biological warfare countermeasures and bioprocess monitoring. In this *tutorial review* we will report on the advances made in bacterial studies, a relatively new and exciting application area for SERS.

Classical methods for typing bacteria

Traditionally, the task of classifying microbes has been performed by a comparison of morphological characteristics or by biochemical tests. More recently genomic analysis has been used as a means of identification or classification, and 16S ribosomal DNA (deoxyribonucleic acid) sequencing is the accepted 'gold' standard used for this task.[1] However, there are drawbacks associated with all of these approaches. Resolution of the method is the main problem and 16S rDNA sequence analysis is only species specific and in order to attain sub-species identification the genome of the organisms generally needs to be known first.

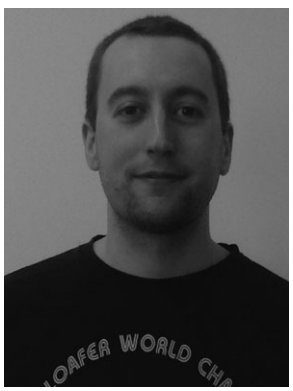
The most popular system of biochemical tests for bacterial identification is called API® (http://www.biomerieux.com/); this is used in routine laboratory analysis, and has been referred to in approximately 2000 publications. With API®, a series of biochemical tests are applied to an organism cultured in the laboratory; the responses of the organism to these tests are then matched against a database of possible results to provide identification.[2] Whilst biochemical detection methods are reliable, they do not always provide conclusive decisions at the species level, whilst strain level characterisation is generally impossible and analysis of environmental isolates uncertain. The process itself is also very time consuming: cell culturing, running the test and analysis of results often require several days.

Therefore conventional microbial typing is inherently slow because of the need to grow the microorganism, and for slow growing organisms like *Mycobacterium tuberculosis* this can take 3–6 weeks.

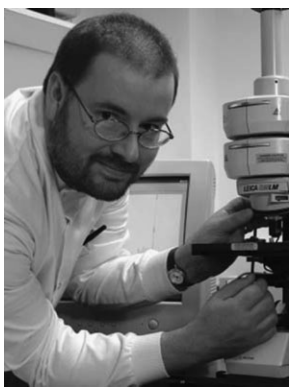
School of Chemistry, Manchester Interdisciplinary Biocentre, University of Manchester, 131 Princess Street, Manchester, UK M1 7DN. E-mail: Roger.Jarvis@manchester.ac.uk; Fax: +44 161 3064519; Tel: +44 161 3065145

Introduction to vibrational spectroscopic characterisation of bacteria

As can be inferred from the above, in just about every area of microbiology there is continuing interest in the rapid and reagentless detection and classification of microorganisms. For example, within clinical laboratories the rapid identification of the causal agent of disease would accelerate targeted prescription and improve epidemiological studies. Other areas that would benefit are biological warfare countermeasures where the immediate detection of the pathogen is essential. In bioprocess monitoring the capability for on-line character-



Dr Roger Jarvis has worked on the application of SERS to bacterial characterisation and identification with multivariate analysis and machine learning. He is employed currently as a PDRA, continuing the theme of SERS with multivariate analysis, in the School of Chemistry, University of Manchester within the Manchester Interdisciplinary Biocentre.



Roy Goodacre is Professor of Biological Chemistry at The University of Manchester. The research group's (http://www.biospec.net/) interests are broadly within bioanalytical chemistry, and in the application of a combination of a variety of modern spectroscopies (including Raman, IR and MS) and advanced chemometrics and machine learning to the explanatory analysis of complex biological systems within a metabolomics and proteomics context.

isation of the microbial manufacturing process is needed for process analytical technology.

The concept of using vibrational spectroscopic techniques to obtain unique, global biochemical 'fingerprints' (often referred to in the recent literature as 'whole-organism fingerprints') from microbial samples is not new. In the early decades of the 20th century, researchers suggested that infrared (IR) absorbance measurements could be used to this effect, although the instrumentation available at the time was not sufficiently advanced to acquire such data. One early study investigated both species and strain (sub-species) level discrimination of bacteria by IR whole-organism fingerprinting.[3] This work showed great potential for sub-species identification, which was demonstrated with the organism *Bacterium tularense*. Since then much work has been undertaken on a variety of bacterial identification problems which utilise IR or its more modern and speedier cousin, Fourier transform-IR (FT-IR) spectroscopy.[4] FT-IR spectroscopy exploits the interferometer, which gives simultaneous chemical information across a broad absorbance range in the form of an interferogram. This is converted to the familiar absorbance spectrum through a mathematical process called the Fourier transform.

More recently, Raman spectroscopy has been applied to the analysis of microorganisms. This technique provides structural information complementary to that obtained by FT-IR absorbance measurements, since IR active vibrational modes are observed from asymmetrical molecular vibrations, whereas Raman bands arise from symmetrical vibrations.[5] In comparison to IR, Raman scattering measurements can be made with greater spatial resolution, allowing highly spatially resolved (sub micron) chemical maps to be generated. This is due to the coupling of an excitation laser to confocal microscope systems and thus the shorter incident light wavelengths employed, that exploit the diffraction limit of light (λ^{-2}). Moreover, water is a weak Raman scatterer, which is beneficial for biological samples that are found naturally in aqueous environments. In addition, because Raman peaks are narrower than IR bands, the technique can offer detailed and more readily interpretable chemical information over a wide range of excitation wavelengths. Although with visible to near infrared (NIR) excitation, biological samples are prone to fluorescence, which can swamp the Raman bands and hence reduce the signal-to-noise levels in the Raman spectrum significantly. Finally, inelastic scattering can exhibit increased sensitivity for specific absorbing species that give rise to resonance enhanced bands across the Raman spectrum, and of course the SERS phenomenon (often complemented by resonance enhancement, the so called double-R SERRS), arising from special properties of roughened metal surfaces, provides huge enhancements with reduced fluorescence backgrounds, that can lend the technique single-molecule sensitivity.[6]

The earliest literature pointing to Raman spectroscopy as a potential tool for biological analyses, in the form of ultraviolet resonance Raman (UVRR) spectroscopy, was published as early as 1974.[7] Resonance Raman enhancements under deep ultra-violet excitation frequencies give rise to sensitive and highly specific quantitative chemical information for chromophoric species, which include the aromatic amino acids, nucleic acids and lipids. The aim in this context is to use UVRR as a structural biology tool, to determine something about the secondary structure of biologically relevant molecules, such as proteins (under the presumption that function is dependent upon these structural motifs in some way), and further how these systems change upon abiotic perturbations that act as control systems in biological networks. A great deal has been achieved in this area,[8] which could be described as microbial systems biology, through the application of Raman spectroscopy methods, and there is currently interest in the application of SERS.

The first experiments aimed at bacterial identification using Raman spectroscopy employed UVRR for single cell analysis, and were reported in the 1980s by Nelson, Sperry and colleagues.[9] Application of UVRR, as with SERS, overcomes the fluorescence problems associated with visible and infrared Raman experiments, because the fluorescence background from UV excitation is recorded beyond the range of the red-shifted Stokes scattered light. However, photochemical damage to the bacterial cells and labile chemical species creates significant boundaries to the widespread adoption of this method. In addition, these early experiments were implemented prior to the widespread use of more advanced multivariate analysis (MVA) methodologies, and therefore only relied on visual inspection of the data to confirm spectroscopic differences that defined different bacterial species. In medicine, however, identification of bacteria to the strain level is necessary (*e.g.*, the accelerated evolution of naturally occurring so called 'superbugs' such as methicillin-resistant *Staphylococcus aureus* (MRSA) or *Clostridium difficile* leads to a need for highly focused taxonomic identification for targeted antibiotic treatments), and the differences observed between UVRR spectra representative of different strains are only subtly quantitative. Therefore, MVA is necessary to form predictive models for bacterial identification, as exemplified for UVRR in [10], based upon patterns of simultaneous quantitative changes observed between different spectra. It is fair to say that the application of MVA to the interpretation of complex vibrational spectroscopic data is gaining popularity for bacterial characterisation, and represents the trade-off that is made between the traditional highly targeted methods of bacterial identification (introduced below/above), which require little data mining, *versus* the combination of whole-organism fingerprinting strategies with advanced chemometrics. For a useful introduction to MVA methods applied to bacterial SERS data see ref. 11.

Deep UVRR technology is, however, expensive in terms of the laser source, optical components and detector. Therefore, in applied studies where the ultimate aim is to deploy technology in the field, one wishes for the sensitivity of deep UVRR with the economy and stability of a visible or NIR Raman system. In the 1990s NIR FT-Raman spectroscopy, which could be used for whole-organism fingerprinting of bacteria, was reported,[12] and qualitative observations were made that suggested information specific to the bacterial cell wall was obtained. However, only more recently was significant progress made in illustrating the capability of Raman spectroscopy for microbial characterisation and discrimination.[13] These authors demonstrated that NIR Raman spectroscopy could be used to characterise bacterial cells in the earliest

stages of colony development. Bacterial microcolonies were analysed after only 6 h of growth, suggesting that Raman is significantly more rapid than biochemical or genetic methods. Additionally, it was shown that Raman could be used to detect phenotypic differences within individual bacterial colonies by collecting a cross section of spectra. Using NIR Raman microspectroscopy, single bacterial cells have also been analysed,[14] and this has been coupled with more complex laser tweezers systems that can trap cells and aid the reduction of fluorescence at NIR wavelengths.[15]

In spite of these developments, Raman spectroscopy studies are still held back by poor sensitivity, with weakly scattering biological samples converting only $\sim$1 in 10^{6-8} incident photons to Raman scattered light. This means that to record a spectrum with good signal-to-noise ratio can take many minutes, and this severely limits Raman spectroscopy for high-throughput bacterial typing. It is therefore no surprise that there is a great deal of interest in using SERS for enhancing the sensitivity of Raman experiments on microorganisms. SERS provides the ultimate in quantitative sensitivity, and gives rise to opportunities for tailored preparation of targeted substrates, the fabrication of which can be an inexpensive and simple process, particularly for colloids. More recent efforts in bacterial SERS in this area have attempted to apply the technology for detecting and identifying vegetative bacteria and bacterial spores that are phenotypically analogous to pathogens that could pose a bio-terrorism threat.[16] There have also been reports of applications within bioprocess monitoring, which requires high sensitivity and quantitative reproducibility as an aid to managing manufacturing processes.[18] Finally, a wealth of literature is emerging which reports the use of SERS for the identification of a range of bacterial species, which has started to gain real momentum in the last few years.[17]

Classification of bacteria by SERS

Clearly, there is a need for new technologies to obtain biochemical information from biological samples, which give excellent sensitivity, maximum chemical information content and quantitatively reproducible signals. When considering the clinical and biosecurity areas, there is ultimately a requirement for an immediate answer, something which is not yet possible with any of the available bacterial typing technology. For this reason many researchers are looking to develop SERS for just this purpose.

The experimental design parameters are such that no standard method for obtaining bacterial SERS spectra has been adopted. This is due largely to the wide range of possibilities available, given the ease of preparing different colloidal substrates and metal films. In 2007 alone, at least 50 primary research articles were published detailing new SERS substrate preparation methods. This rate of development has been fairly consistent over the last decade, meaning that it can often be difficult to draw a line under the advancement of SERS experimental design with respect to bacterial studies, and nor should we!

However, it is clear that bacterial SERS studies have most often utilised colloidal substrates, with simple mixing followed by drying on to an appropriate Raman spectroscopy substrate, prior to spectral acquisition. Interestingly, an early bacterial SERS study used an *in vitro* reduction of silver with sodium borohydride to encapsulate bacteria in colloidal particles.[19] In the preparation of any metal nanoparticles for SERS the main variables to be controlled are the type of colloid used and the aggregating agent, with the general rule of thumb being 'if it works, use it!' In Fig. 1, the scanning electron micrograph (SEM) image shows a typical matrix of cultured *Escherichia coli* bacteria measuring $\sim$1–1.5 μm by 0.5 μm, with citrate reduced colloidal silver deposits (prepared following [20]). In this case the mixture was deposited on to an aluminium SEM stub, as opposed to a more conventional calcium fluoride or fused silica slide, and bacterial SERS spectra were acquired from this matrix to ensure that the conditions were SERS active.

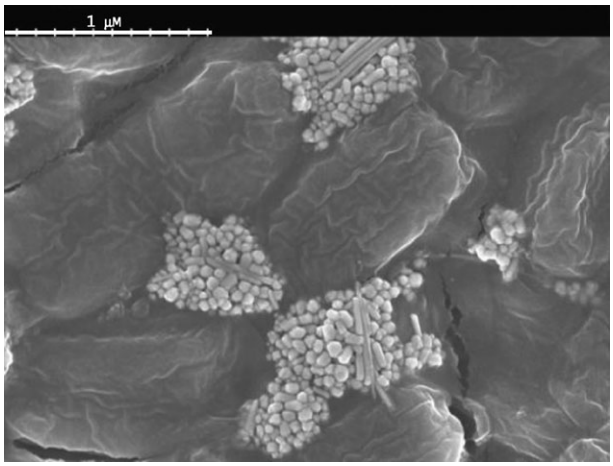


Fig. 1 SEM image of *Escherichia coli* cells and aggregated citrate reduced colloidal silver.

The main problem encountered with this approach is that if one uses a low numerical aperture (NA) objective lens through which to focus incident laser light and achieve maximum energy density at the sample, the illumination volume will often not be sufficient to encompass a whole bacterial cell and adjacent colloid. This leads to major problems with obtaining reproducible single-shot point spectra at high magnification with this particular approach. Therefore, we used a simple method of averaging SERS spectra (Fig. 2) after acquiring multiple (n = 50, each collect was 10 s) samples, and were able to derive reproducible multivariate statistical models to identify different strains of *E. coli* bacteria and also different species of bacteria recovered from patients with urinary tract infections (UTIs). The main drawback is that the speed of acquiring data using SERS is lost when multiple spectra are measured to represent a single sample

Further studies, including our own, have compromised to some extent on maximum SERS enhancements by using a higher NA objective lens, giving an increased collection volume to obtain an average SERS profile from a sample directly.[21] This is perhaps the preferred sampling option, since even under high magnification with optical microscopes it is virtually impossible to target a bacterial cell–colloid junction. Therefore it may be better to accept lower spatial precision for

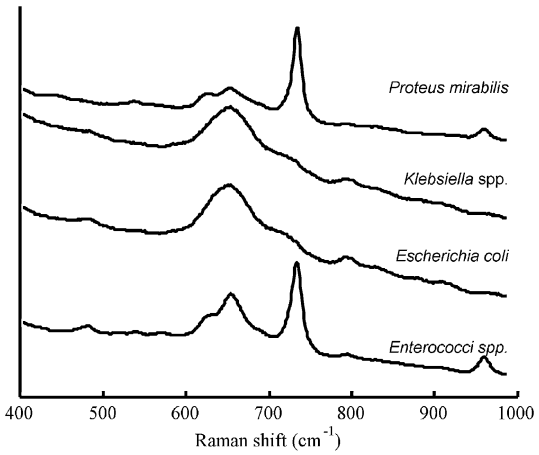


Fig. 2 Example SERS spectra of bacteria isolated from patients with UTIs.

more globally representative data. A more expensive alternative, that requires lengthier sample preparation and data acquisition time, is to use a Raman instrument coupled to an electron microscope.[22]

One of the fascinating aspects of bacterial SERS is the radical difference in chemical information observed when experimental parameters are altered. For instance, the spectra shown in Fig. 2 were obtained using near infrared (NIR) excitation at 785 nm, with a citrate reduced silver colloid and NaCl aggregating agent. Compare the spectrum of *E. coli* in this figure to that shown in Fig. 3, which is another SERS spectrum of *E. coli* (a laboratory strain UB5201), this time acquired with green excitation (532 nm) and a borohydride reduced silver colloid. The spectra are entirely different, despite the fact that the cells have very similar surface chemistries. Given that the spectrum of *E. coli* using borohydride reduced colloid appears to have far greater information content (although some features in Fig. 2 are obscured due to scaling), it would not be imprudent to suggest that this method gives far better SERS. However, the *Bacillus subtilis* (type strain B0014^{T}) spectrum in Fig. 3 is virtually indistinguishable from its counterpart, despite the fact that these organisms

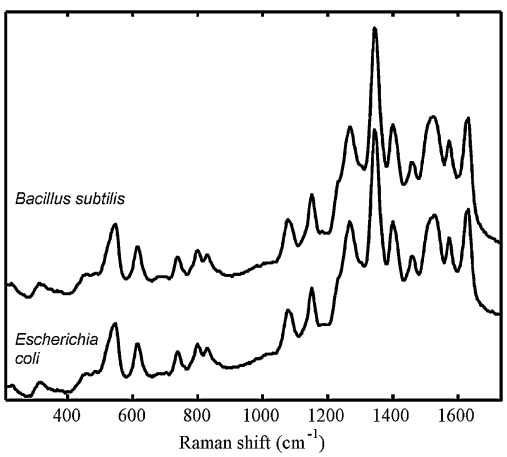


Fig. 3 SERS spectra of *E. coli* and *B. subtilis*, acquired using 532 nm exciting light and a borohydride reduced silver colloid.

have entirely diverse surface chemistries. Therefore, in a bacterial identification study one would still favour the citrate reduced silver method.

Another interesting development in the field of enhanced Raman spectroscopy, that has already shown great promise for bacterial studies, is tip enhanced Raman spectroscopy (TERS). With this approach, an atomic force microscopy (AFM) experiment is performed using a SERS active tip, that provides detailed spatial and chemical information simultaneously from the sample. This method allows the opportunity to obtain spatially resolved chemical images below the diffraction limit of light, and has already been demonstrated in mapping the surface of single *Staphylococcus epidermidis* (ATCC 35984) cells.[23]

It is clear that the field is at the stage (there are nearly 100 bacterial SERS articles, using the search term 'SERS' and 'bacter*' or 'microb*' in Web of Knowledge; http://wos.mimas.ac.uk/) where SERS offers a very valuable and powerful additional approach in the 'whole-organism fingerprinting' toolbox for the identification of bacteria to the strain level, and that these measurements can be made reproducible under controlled experimental conditions. The challenge now is to define some standard operating procedures (substrates, aggregating agents, excitation frequencies, sampling methodologies) that could be employed on a widespread basis, that are viable for the direct analysis of environmental or medical samples.

Identification of bacteria in clinical microbiology

The infection of humans by pathogenic bacteria is not uncommon and in otherwise healthy individuals very easily treated by antibiotics. However, there is a major concern about the continuing effectiveness of broad-spectrum antibiotics, which for a variety of reasons are having a diminished effect upon the treatment of certain infections. This leads to a need for the improvement of best prescription practices, which can be achieved if an accurate and rapid identification of the bacteria causing infection is made. This would make it possible to provide a targeted treatment of the illness, which would involve using an antibiotic proven to be more effective against a certain microorganism, thus reducing the likelihood of resistant strains emerging in the future, or at least increasing the effective lifetime of certain treatments. To date, only the identification of UTI bacteria (described above) has been addressed using SERS,[17] and there are many other disease causing bacteria that could be addressed, especially those where the requirement to cultivate the organism could be removed by the sensitive analysis offered by SERS.

An additional area where SERS could be very effective is for the identification of bacterial pathogens causing secondary opportunistic infections in very sick patients. In an already weakened individual, the immune system may be unable to overcome an infection even with the aid of suitable antibiotic treatments, unless those are highly targeted. Also, it would be preferable to identify a pathogen before the patient starts to exhibit symptoms of illness, which may mean analysing biomass concentrations as low as 1 cfu mL^{-1}. Given that both Raman spectroscopy and SERS have been shown to be

sensitive to this level,[24] it would be an ideal candidate for the direct screening of patient blood and urine.

Detection of biological warfare agents

There is currently great interest in methods by which biological warfare agents can be detected rapidly and safely (*i.e.*, remotely) in the field. In such a scenario bacterial identification is needed immediately and one needs to avoid culturing the agent, and therefore SERS could be ideally placed to provide an analysis tool for such situations.

In 2001 anthrax was used for bioterrorism purposes and was deployed in letters in Florida, resulting in inhalation anthrax which is much more virulent compared to the cutaneous or intestinal disease.[25] The causal agent is *Bacillus anthracis*, a notorious bacterium that belongs to a genus whose members protect themselves from extreme changes in environmental conditions by developing a tough protein coat around the genetic material in the cell to form a spore, which can survive dry conditions and extremes of temperature and pH. Since these spores are hardy they are ideal for deployment as a biowarfare agent and their detection in aerosols is also necessary.

The identification of spores has been the subject of great interest over many years, and methods including fluorescence, flow cytometry, pyrolysis mass spectrometry (MS) and electrospray MS are amongst those which have been applied in order to detect bacterial spores. Common to all of these studies is the detection of the dipicolinic acid (DPA; pyridine-2,6-dicarboxylic acid) biomarker. DPA is a compound present in all spores from the *Bacillus* genus (reported range 1–14%). Therefore, if this compound is detected it is very likely that a spore is present. The most recent work on the detection of bacterial spores demonstrates correlations between the DPA SERS spectrum and SERS spectra of spores.[26–28] Fig. 4 shows SERS spectra of *Bacillus* spores, as reported in ref. 21, which correlate very well with the SERS spectrum of pure DPA. However, DPA containing bacterial spores from non-lethal species are also present in the environment. Therefore, one still needs to demonstrate that SERS spectra of bacterial spores from different species and strains within a species can be used to identify that the sample is a bacterial spore, and also specify which bacterial species it belongs to. So far the latter has been attempted using vegetative bacilli.[21,29]

Metabolic profiling of microorganisms

One topic in the life sciences that is gaining increasing interest is the understanding of how a cell works at the molecular level. As microorganisms can be grown under highly controlled conditions in chemostats they are excellent models for understanding complex biochemical processes and their interactions. In a Systems Biology context, this means developing computer models that embody isolated or global biochemical interactions (usually as metabolic networks), that can be parameterised using quantitative data acquired either from the organism or input according to estimates.[30] These models have the potential to enable many important applications to be investigated, such as predictive modelling of biochemical network behaviour, when a notional biotic or abiotic perturbation is imposed upon the simulation. This leads to the possibility of understanding drug metabolism which would aid rapid development of new treatments. Another significant area of application is in using these models to predict changes in global metabolism resulting from the removal of a single gene from an organism's genome, and so determine the function of that gene, so called Functional Genomics.[31] Given that only ~40% of genes identified through genome sequencing projects have a known function, there is much interest in this area. In addition, there is a possibility for the theoretical system to be extended to more complex cases, once proven for simple organisms such as bacteria, since certain cellular functions such as ATP synthesis, for instance, operate in an identical way across all cell types.

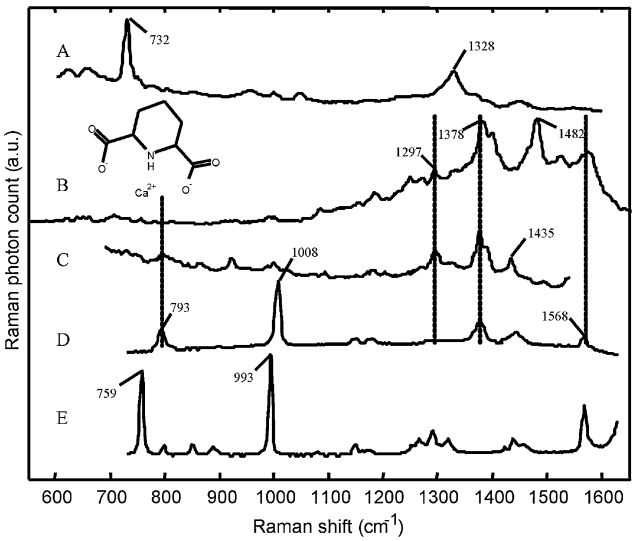


Fig. 4 Reproduced from ref. 21. SERS spectra of *B. subtilis* B0014^T acquired with 785 nm excitation; (A) vegetative cells; (B) sporulated cells acquired using a Raman probe coupled to a SEM; (C) sporulated cells acquired with a Raman microscope; (D) SERS spectrum of DPA; (E) conventional Raman spectrum of DPA. The vertical dotted lines indicate complementary Raman bands within the spore SERS spectra and the DPA SERS spectrum.

One of the main challenges in Systems Biology is obtaining accurate quantitative data with which to parameterise models, and since many of the models are at the level of interconnecting metabolic pathways (or networks) an essential part of this is metabolic profiling.[32,33] In order to identify and quantify the many metabolites that are present in a cell, techniques are required which have exquisite sensitivities and wide dynamic ranges. Currently the most popular methods employed are MS and NMR. The drawback with MS is that complex sample preparation steps are usually required, and effects such as retention drift (when coupled to chromatography), ion suppression and adduct synthesis create significant challenges for the analyst. Whilst sample preparation for NMR is more straightforward, it does suffer from poor sensitivity. Consequently, the interesting possibility of applying SERS in this field arises, and given we know that the limits of sensitivity rival MS, the question is whether or not the method is sufficiently quantitatively reproducible? As a step towards this we have generated preliminary SERS data[11] from the 'metabolic footprint' of a range of yeast (*Saccharomyces cerevisiae*)

single knockout mutants. The application of simple MVA methods to these data showed that differences in the biochemistry of the metabolic footprint of different yeast mutants were detected by SERS. The SERS spectra in this case could not be deconvolved into component metabolites and this may be achieved by coupling SERS to a separation method, in order to make a proper comparison to current technologies.

Bioprocess monitoring

The control of a bioprocess is essential for product yield optimization, and it is imperative that the concentration of the fermentation product is quantified accurately.[34] Given that Raman spectroscopy has historically been used to monitor the reaction of chemical processes, it was only a matter of time before this approach was used to analyse bioprocesses. Since chemical reactions involve only a few chemical species the Raman vibrational modes are more readily attributable to substrates and products. However, biological processes are much more complex and rarely can Raman bands be related directly to bioproduct formation. In addition, for low concentration products in a complex mixture, the signal from the analyte of interest is often masked because of weak Raman scattering, and this has perhaps discouraged analysts from taking this approach. However, SERS provides an opportunity for off-line and at-line monitoring of bioprocesses which is sensitive both for the detection and quantification of low yield primary and secondary metabolites, with the possibility of on-line analysis using suitably inert substrates, an important consideration since colloidal silver is a well known biocide.

Initial work conducted in our laboratories has investigated the production of penicillin using SERS.[18] This industrially important bioprocess utilises the *Penicillium chrysogenum* fermentation to yield penicillin G as the major secondary metabolite of commercial interest. With conventional Raman spectroscopy using NIR 785 nm excitation, the Raman spectrum of penicillin G at high concentrations is dominated by the resonance enhancement of the aromatic ring vibration at 1005 cm^{-1}. Contrastingly, the SERS penicillin G spectrum, when acquired at 785 nm, reveals many more bands, with a much improved signal to noise and significantly reduced fluorescence. With respect to the quantification of penicillin G, we have shown that Raman spectroscopy could be used to quantify the amount of penicillin present in broths when relatively high levels of penicillin were analysed (>50 mM). Encouragingly, using simple integration under SERS bands, excellent quantification of penicillin G from considerably lower concentrations of the antibiotic was achieved.

Conclusions

Vibrational spectroscopies meet the requirement for rapid, accurate and automated methods for the identification and characterisation of microorganisms. These techniques require only minimal sample preparation, permit the automatic analysis of many serial samples with negligible reagent costs, allow for rapid characterisation against a stable database, are easy to use, and can be operated under the control of a laptop computer. However, it is the enhanced methods of Raman spectroscopy, including SERS, which have the potential to provide truly rapid analysis of biological samples without the need for cell culture, and could provide major benefits in many areas of microbiology.

Acknowledgements

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